ORIGINAL ARTICLE 10.1111/j.1469-0691.2007.01890.x

Use of a multiplex PCR-based reverse line blot (mPCR/RLB) hybridisation assay for the rapid identification of bacterial pathogens

Y. Wang[1], F. Kong[2], G. L. Gilbert[2], M. Brown[2], W. Gao[1], S. Yu[1] and Y. Yang[1,3]

[1]Beijing Children's Hospital Affiliated to Capital Medical University, Beijing, China, [2]Centre for Infectious Diseases and Microbiology (CIDM), Institute of Clinical Pathology and Medical Research (ICPMR), Westmead, New South Wales, Australia and [3]Children's Hospital of Fudan University, Shanghai, China

ABSTRACT

The aim of this study was to develop a sensitive and reliable method for the molecular identification of pathogenic bacteria. A multiplex PCR-based reverse line blot (mPCR/RLB) hybridisation assay was developed and evaluated for the rapid identification of 24 systemic and respiratory bacterial pathogens in routine diagnosis. All species-specific probes designed for the RLB hybridised with amplified DNA only from the corresponding species. Sensitivity limits of the mPCR/RLB assay varied among the 24 target organisms from 0.05 pg to 0.5 ng of genomic DNA. The sensitivity of the assay was 2×10^2 CFU/mL for *Streptococcus pneumoniae* and 6×10^2 CFU/mL for *Escherichia coli*. The specificity of each probe was tested against 24 species. There were no cross-reactions among any of the 43 probes. The mPCR/RLB assay appeared to be a useful alternative tool for the molecular identification of common pathogens.

Keywords Bacterial pathogens, diagnosis, hybridisation assay, identification, multiplex PCR-based reverse line blot (mPCR/RLB), probes

Original Submission: 18 October 2006; **Revised Submission:** 9 May 2007; **Accepted:** 10 September 2007

Clin Microbiol Infect 2008; **14**: 155–160

INTRODUCTION

Bacteraemia and sepsis are potentially life-threatening conditions in which rapid identification of the causative agent is essential to guide the most appropriate antimicrobial therapy [1,2]. Infection with different agents can cause similar signs and symptoms that are often indistinguishable clinically [3].

Cultivation of blood in commercially available blood culture bottles is the reference standard for detection of bacteraemia, but usually requires >72 h for final identification of the infecting bacteria. In addition, blood cultures often remain negative, especially if use of antimicrobial agents in the community is widespread. Rapid identification of the causative agent in cases of septicaemia contributes to better patient outcome, and can also reduce unnecessary use of broad-spectrum antibiotics, thereby helping to prevent the emergence of antibiotic resistance. Based on a well-established multiplex PCR-based reverse line blot (mPCR/RLB) hybridisation assay protocol [4], the present study describes the development of an assay that allows the simultaneous identification of up to 24 common, clinically significant pathogens.

MATERIALS AND METHODS

Bacterial isolates

Representative strains of 24 bacterial species used to develop the assay, and their sources, are shown in Table 1. Clinical isolates (n = 100) from the diagnostic laboratory at the Centre for Infectious Diseases and Microbiology (Institute of Clinical Pathology and Medical Research (ICPMR), Westmead, New South Wales, Australia) were used to evaluate the assay. All clinical isolates were identified according to standard conventional methods.

DNA extraction

Isolates (*c.* 5 CFU) from horse blood, chocolate or buffered charcoal yeast extract agar (BCYE) plate cultures, as appropriate, were suspended in 100 μL of digestion buffer (10 mM

Corresponding author and reprint requests: Y. Yang, Beijing Children's Hospital Affiliated to Capital Medical University, Nan Lishi Road 56, Beijing 100045, China
E-mail: yyh66@vip.sina.com

Table 1. Bacterial species and isolates used to develop and evaluate the multiplex PCR-based reverse line blot (mPCR/RLB) assay, together with the DNA detection limits for each species

Species	Strain ID number	DNA concentration (mg/L)	Detection limit of mPCR/RLB[a]	Genome size (bp)	Genome copies/mL	Genome copies/5 μL	No. of clinical isolates
Staphylococcus aureus	ATCC 25923	43	0.5 pg	2 902 619	3.14×10^4	1.57×10^2	9
Staphylococcus epidermidis	ATCC 12228	31.5	0.5 pg	2 499 279	3.65×10^4	1.83×10^2	2
Staphylococcus hominis	ATCC 27844	22.4	0.5 pg	2 902 619	3.14×10^4	1.57×10^2	3
Staphylococcus haemolyticus	ATCC 29970	5.5	0.5 pg	2 685 015	3.40×10^4	1.7×10^2	3
Staphylococcus saprophyticus	ATCC 15305	70	5 pg	2 516 575	3.62×10^5	1.81×10^3	1
Streptococcus pneumoniae	SSI serotype 14	2.9	0.5 pg	2 046 115	4.46×10^4	2.23×10^2	7
Streptococcus pyogenes	ATCC 19615	8.9	0.5 pg	1 852 441	4.92×10^4	2.46×10^2	3
Streptococcus agalactiae	ATCC BAA-22	23.4	5 pg	2 160 267	4.22×10^5	2.11×10^3	5
Neisseria meningitidis	ATCC 13102	38.5	5 pg	2 194 961	4.16×10^5	2.08×10^3	2
Moraxella catarrhalis	Clinical isolate[b]	64.4	5 pg[d]				5
Haemophilus influenzae	ATCC 10211	9.4	5 pg	1 830 138	4.98×10^5	2.49×10^3	10
H. influenzae type b	PHI/UK	82	50 pg	1 830 138	4.98×10^6	2.49×10^4	3
Bordetella pertussis	Clinical isolate[b]	56.9	5 pg	4 086 189	2.23×10^5	1.11×10^3	4
Escherichia coli	ATCC 25922	47.5	5 pg	5 498 450	1.66×10^5	8.3×10^2	6
Pseudomonas aeruginosa	ATCC 27853	75	50 pg	6 264 404	1.46×10^6	7.3×10^3	5
Klebsiella pneumoniae	ATCC 13883	60.5	0.5 pg[c]				5
Legionella pneumophila	ATCC 33152	45	5 pg	3 345 687	2.73×10^5	1.37×10^3	6
Listeria monocytogenes	ATCC 19112	11.4	0.05 pg	2 944 528	3.1×10^3	1.55×10^1	2
Enterococcus faecalis	ATCC 29212	28.9	50 pg	3 218 031	2.83×10^6	1.42×10^4	3
Acinetobacter spp.	ATCC 42627	57.4	5 pg	3 598 621	2.53×10^5	1.27×10^3	2
Bacteroides fragilis	ATCC 25285	39.4	50 pg	5 205 140	1.75×10^5	8.75×10^2	1
Mycobacterium tuberculosis	ATCC 27254-H37RvD[d]	42.5	50 pg	4 411 532	2.07×10^6	1.03×10^4	4
Mycoplasma pneumoniae	TW183-ATCC VR-2282 D[d]	18	0.5 ng	4 345 492	2.1×10^7	1.05×10^5	5
Chlamydia pneumoniae	ATCC 15531[b]	85.1	0.5 ng	1 225 935	7.44×10^7	3.72×10^5	2

ATCC, American Type Culture Collection; SSI, Statens Serum Institute, Denmark; PHI/UK, Public Health Institute/UK.
[a]As determined by dilution series (see text for details).
[b]Clinical isolates were from the Centre for Infectious Diseases and Microbiology (Institute of Clinical Pathology and Medical Research, Westmead, New South Wales, Australia) and were identified by standard microbiological methods.
[c]Currently, there are no published genome sequences for *Moraxella catarrhalis* and *Klebsiella pneumoniae*, so calculation of genome equivalents was not possible.
[d]Genomic DNA prepared previously and stored in our laboratories.

Tris-HCl, pH 8.0, Triton X-100 0.45% v/v, Tween-20 0.45% v/v), heated at 100°C for 10 min, and then cooled on ice. After centrifugation at 16 100 *g* for 1–2 min, 400 μL of 0.5 × TE buffer (1 × TE is 10 mM Tris, 1 mM EDTA, pH 8.0) was added to each tube. The tubes were re-centrifuged and 5 μL of supernatant was used as the DNA template in subsequent amplification experiments.

Primer and probe design

Species-specific primers and probes were designed, based on a well-established strategy [4], to allow simultaneous amplification in a mPCR. To minimise cross-hybridisation, species-specific primers and probes (Table S1, see Supplementary material) were identified by comparison of sequences in GenBank using the Pileup and Pretty programs in the Multiple Sequence Analysis program group provided in Biomanager (http://biomanager.angis.org.au/) by the Australian National Genomic Information Service (ANGIS). The oligonucleotide sequences were also analysed for possible self-interactions and hairpin loop structures, but no significant theoretical interactions were identified. To test for theoretical specificity, all the oligonucleotides used were aligned with the sequence databases of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLASTn). Primers were labelled at the 5′-end with biotin to allow PCR products to be detected by hybridisation with a streptavidin–peroxidase substrate in the RLB assay. All probes were labelled at the 5′-end with an amine group to facilitate covalent linkage to nylon membranes and to allow membranes to be stripped and reused repeatedly.

mPCR

The mPCR mixture containing the 22 primer-pairs was as follows [4]: 5 μL DNA extract, 0.25 μL each forward (50 pmol/μL) and reverse (50 pmol/μL) primer, 1.25 μL dNTPs mix (2.5 mM each dNTP), 2.5 μL 10 × PCR buffer, 4.5 mM $MgCl_2$ (final concentration), 0.5 U Hotstar *Taq* DNA polymerase (Qiagen, Shanghai, China) and water to 25 μL. mPCR was performed in single tubes and comprised 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by 72°C for 10 min.

RLB hybridisation

The RLB hybridisation assay was performed as described previously [4], except that the streptavidin–peroxidase conjugate (Roche Diagnostics, Basel, Switzerland) was diluted 3:5000 in 2 × SSPE (0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.7) with SDS 0.5% w/v, and the time of exposure to X-ray film (Hyperfilm; Amersham, Piscataway, NJ, USA) was 10 min. The oligonucleotide probes were tested at several concentrations, ranging from 0.6 to 3 pmol/μL, to determine the optimal concentration of the probes for RLB.

Determination of analytical sensitivity and specificity

For specificity studies, DNA was extracted from reference strains (Table 1) using a DNA Miniprep Extraction kit (Sigma, St Louis, MO, USA) according to the manufacturer's instructions, and was tested against all primer sets. Serial ten-fold dilutions of genomic DNA were prepared in distilled water. In

addition, serial ten-fold dilutions (starting at 10^5 CFU/mL) of suspensions of cultures of *Escherichia coli* ATCC 25922 and *Streptococcus pneumoniae* SSI 14 were prepared in physiological saline. Crude DNA was extracted from 1 mL of these suspensions by heating at 100°C for 10 min.

Sequencing and sequence analysis

Sequencing was performed as described previously [5]. All programs used in the study were provided by BioManager (ANGIS), including Work Bench for sequence file management, BESTFIT for two sequence comparison, PILEUP and PRETTY in the multiple sequence analysis program groups, and BLASTn for sequence searches.

RESULTS

Selection of primers and probes

In total, 48 primers 18–26 bp in length were designed, with melting temperatures (T_m) of 59.5–79.3°C and amplicon sizes in the range 94–371 bp (Table S1). Forty-two different oligonucleotide probes directed against specific bacteria were designed. In addition, a universal probe targeting the 16S rRNA gene was used as a control. To allow optimal hybridisation under the same conditions, probes were also designed with specific physical characteristics, i.e., a length of 17–28 bp and a T_m of 58.6–70.7°C. Initial evaluation experiments revealed that the optimal probe concentration was 3 pmol/μL for the MccopBAp, BpporinAp, HigyrBAp, LpmipSp, MpS-Sap and MpS-SSp oligoprobes (Table S1), and 2 pmol/μL for other probes.

Identification of a range of bacteria

All species-specific probes designed for RLB hybridised only with amplified DNA from the corresponding species. Fig. 1 illustrates the hybridisation patterns obtained with 24 reference strains of bacteria. Detection limits of the mPCR/RLB assay varied among the 24 target organisms, from 0.05 pg to 0.5 ng of genomic DNA, or *c.* 15-10^6

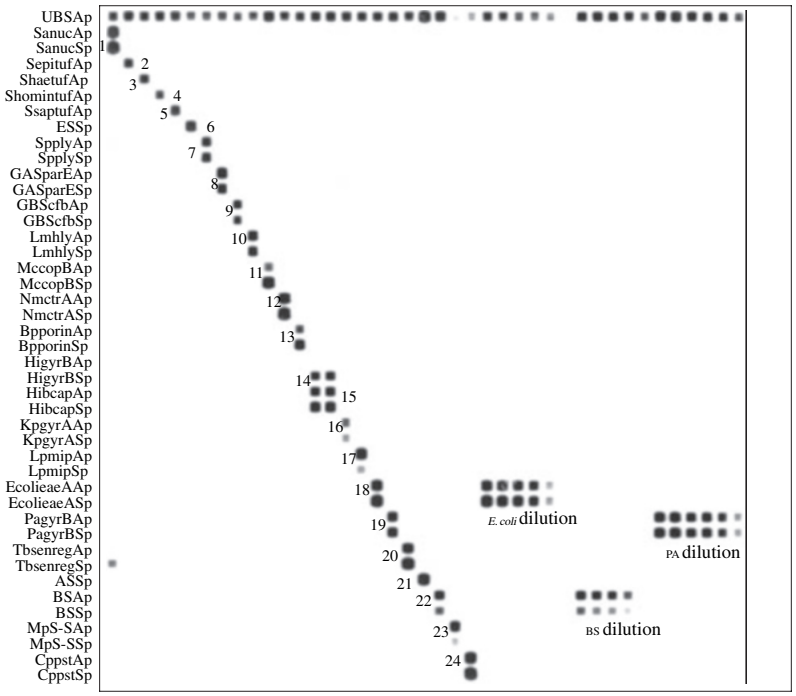

Fig. 1. Detection of 24 bacterial reference strains using the multiplex PCR-based reverse line blot assay. DNA dilutions were as follows: *Escherichia coli* (500 ng - 5 pg), *Bacteroides* spp. (BS) (500 ng - 50 pg), *Pseudomonas aeruginosa* (PA) (5 μg - 50 pg). Probes are indicated on the *y*-axis, and bacteria are indicated on the *x*-axis. Numbers refer to reference strains of the following species: 1, *Staphylococcus aureus*; 2, *Staphylococcus epidermidis*; 3, *Staphylococcus haemolyticus*; 4, *Staphylococcus hominis*; 5, *Staphylococcus saprophyticus*; 6, *Enterococcus faecalis*; 7, *Streptococcus pneumoniae*; 8, *Streptococcus pyogenes*; 9, *Streptococcus agalactiae*; 10, *Listeria monocytogenes*; 11, *Moraxella catarrhalis*; 12, *Neisseria meningitidis*; 13, *Bordetella pertussis*; 14, *Haemophilus influenzae*; 15, *H. influenzae* type b; 16, *Klebsiella pneumonia*; 17, *Legionella pneumophila*; 18, *E. coli*; 19, *P. aeruginosa*; 20, *Mycobacterium tuberculosis*; 21, *Acinetobacter* spp.; 22, *Bacteroides* spp.; 23, *Mycoplasma pneumoniae*; and 24, *Chlamydia pneumonia*.

genome equivalents in 5 μL (Table 1). The sensitivity of the assay was 2×10^2 CFU/mL for *S. pneumoniae* and 6×10^2 CFU/mL for *E. coli* (Fig. 1).

When 100 clinical isolates were tested using the mPCR/RLB assay, the original identities of all the clinical isolates were confirmed by RLB with distinct and unambiguous hybridisation patterns. There were no cross-reactions among any of the 43 probes, each of which hybridised only with the corresponding species among the 24 reference strains and 100 clinical strains.

Sequence analysis results

Fifteen *Haemophilus influenzae* isolates, including four isolates of *H. influenzae* type b, produced strong hybridisation signals with the species-specific forward probe. However, amplification products from seven isolates produced weak hybridisation signals, and two isolates produced a negative hybridisation signal with the reverse probe. Sequence analysis showed that weak signals were associated with a single base mutation and negative results with two base mutations. A newly identified *gyrB* sequence with base mutations was deposited in GenBank with accession number U32738.

DISCUSSION

The most widely used method for the identification of common bacterial infections involves conventional microbiological culture [6]. Blood culture is the standard reference method for detection of bacteraemia, but requires incubation for 8–24 h, and for up to 72 h for final identification [6]. If antimicrobial therapy has already been started, blood cultures are likely to be negative, even in cases of severe sepsis. In China, 98% of children with common colds and other upper respiratory infections are given antimicrobial agents [7]. For the clinician, rapid identification of the causative agent in blood cultures or body fluids contributes to a better outcome. In addition, unnecessary use of broad-spectrum antibiotics and the risk of emergence of antibiotic resistance are reduced because the identity of the agent provides clues to its susceptibility. Although molecular identification techniques, e.g., PCR, have been developed, especially for slow-growing or fastidious bacteria, it is only practical to perform a limited number of individual PCR assays [8]. A molecular diagnostic tool that allows rapid simultaneous identification of many possible aetiological agents is needed to enable appropriate measures to be instituted more rapidly and to reduce morbidity and mortality, especially in children and immunocompromised patients.

In order to achieve rapid identification of the causative agents of bacteraemia and pneumonia, the present study describes a novel, sensitive and simple mPCR/RLB assay, based on the use of primers and probes that recognise conserved species-specific sequences of bacterial genes encoding essential molecules [4]. The use of this mPCR/RLB assay facilitates the identification of multiple aetiological agents more easily than either multiple, individual species-specific PCRs or a PCR using a 'universal' bacterial target followed by sequencing [9]. The probes used in the present study were 17–28 bp in length and targeted the 5′-ends of the amplicons obtained by PCR. Previous studies have described the use of a 'universal' eubacterial 16S rDNA PCR to amplify DNA fragments of 360–380 bp, depending on the bacterial species, at the 5′-end of the 16S rRNA gene, followed by sequencing or restriction enzyme analysis to identify the species [9]. However, the 16S rDNA region flanked by these eubacterial primers cannot differentiate reliably among all relevant eubacterial species [10]. Therefore, the present study targeted individual species-specific genes for amplification, while corresponding species-specific probes were used in the mPCR/RLB assay to differentiate among amplicons from different species and to increase the specificity. All 100 clinical isolates tested gave the expected results within *c.* 7 h of completion of the mPCR. However, although there were no cross-reactions among the probes used for identification of individual species, there were several species for which the results were discrepant between the forward and reverse probes, e.g., *H. influenzae*, *Mycoplasma pneumoniae* and *Legionella pneumophila*. For the *H. influenzae* probes (20–24 bp), the 5′-end of the region amplified using the primers designed by Roth *et al.* [11] was targeted. In some cases, the reverse probes gave weak or negative signals because of minor variations in the target sequence. However, all isolates were identified

reliably by the forward probes. It is assumed that there was a similar explanation for discrepancies between the two probes for other individual bacterial species that gave weak or false-negative signals.

Analysis of amplicons in the RLB chemiluminescent hybridisation assay is 100-fold more sensitive and faster than the use of ethidium bromide-stained agarose gels [9], and allows detection of PCR products not visualised by fluorescent dye staining [12]. Although the development of mPCR/RLB assays, which requires expertise in the design of primers and probes [4], is relatively expensive and limited to research or specialised diagnostic laboratories, the marginal cost of each test, once developed, is modest, and assays could be produced as kits for use in the routine diagnostic laboratory. A particular advantage of mPCR/RLB is that the membranes can also be stripped and re-used up to 20 times without substantial loss of sensitivity [13].

Molecular methods do not depend on the presence of viable or growing bacteria, and so are particularly suited to species that cannot be cultured readily by routine methods, or that have been damaged by exposure to antibiotics [6], as has been demonstrated previously by the use of broad-range PCR amplification directly with clinical specimens [14]. Further evaluation of the mPCR/RLB method is warranted with respect to the direct detection of bacterial DNA in clinical samples, e.g., cerebrospinal fluid specimens, that meet cytological and biochemical criteria for infection despite a negative culture result.

Finally, the mPCR/RLB method is potentially suitable for use with large numbers of specimens, as it can analyse 43 clinical samples simultaneously, does not require expensive instrumentation, and provides rapid results when compared with conventional culture and alternative molecular methods (e.g., sequencing and restriction fragment length polymorphism analysis). Thus, this method could become a powerful and reliable tool for the identification of common pathogens [4]. Future research should focus on the direct identification of bacterial DNA in samples that are suspected on clinical evidence to contain pathogens, with particular reference to samples that yield negative culture results.

ACKNOWLEDGEMENTS

The project was funded by the Ministry of Science and Technology of China (No. 2003BA712A11-20), Bureau of Science and Technology of Beijing (No. Y0204004040131), and Young Award of Bureau Science and Technology of Beijing (No. 2004B02). We are also grateful to Wyeth Pharmaceuticals Inc. for their support in the preparation of this paper. No information has been provided by the authors concerning the existence or absence of conflicting or dual interests.

SUPPLEMENTARY MATERIAL

The following Supplementary material is available for this article online at http://www.blackwell-synergy.com:

Table S1. Primers and probes used in this study

REFERENCES

1. Wiessner WH, Casey LC, Zbilut JP. Treatment of sepsis and septic shock: a review. *Heart Lung* 1995; **24**: 380–392.
2. Nicholls TM, Morgan AS, Morris AJ. Nosocomial blood stream infection in Auckland healthcare hospitals. *NZ Med J* 2000; **113**: 96–98.
3. Putto A, Ruuskanen O, Meurman O. Fever in respiratory virus infections. *Am J Dis Child* 1986; **140**: 1159–1163.
4. Kong F, Gilbert GL. Multiplex PCR-based reverse line blot hybridization assay (mPCR/RLB)—a practical epidemiological and diagnostic tool. *Nat Protocols* 2007; **1**: 2668–2680.
5. Kong F, Gowan S, Martin D *et al*. Serotype identification of group B streptococci by PCR and sequencing. *J Clin Microbiol* 2002; **40**: 216–226.
6. van Haeften R, Palladino S, Kay I *et al*. A quantitative LightCycler PCR to detect *Streptococcus pneumoniae* in blood and CSF. *Diagn Microbiol Infect Dis* 2003; **47**: 407–414.
7. Yang YH, Jiang ZF, Chen XN *et al*. Countercurrent immunoelectrophoresis for diagnosis of acute bacterial pneumonia in Chinese children. *Chin Med J (Engl)* 1993; **106**: 105–109.
8. Khanna M, Fan J, Pehler-Harrington K *et al*. The pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae*, *Chlamydia* (*Chlamydophila*) *pneumoniae*, *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis*, and its real-time counterpart. *J Clin Microbiol* 2005; **43**: 565–571.
9. Pille F, Martens A, Schouls LM *et al*. Detection of bacterial DNA in synovial fluid from horses with infectious synovitis. *Res Vet Sci* 2004; **77**: 189–195.
10. Fukushima M, Kakinuma K, Kawaguchi R. Phylogenetic analysis of *Salmonella*, *Shigella*, and *Escherichia coli* strains on the basis of the *gyrB* gene sequence. *J Clin Microbiol* 2002; **40**: 2779–2785.
11. Roth SB, Jalava J, Ruuskanen O *et al*. Use of an oligonucleotide array for laboratory diagnosis of bacteria responsible for acute upper respiratory infections. *J Clin Microbiol* 2004; **42**: 4268–4274.
12. Mariani BD, Levine MJ, Booth RE *et al*. Development of a novel, rapid processing protocol for polymerase chain

reaction-based detection of bacterial infections in synovial fluids. *Mol Biotechnol* 1995; **4**: 227–237.
13. Kamerbeek J, Schouls L, Kolk A *et al*. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol* 1997; **35**: 907–914.
14. Bosshard PP, Kronenberg A, Zbinden R *et al*. Etiologic diagnosis of infective endocarditis by broad-range polymerase chain reaction: a 3-year experience. *Clin Infect Dis* 2003; **37**: 167–172.
15. Costa AM, Kay I, Palladino S. Rapid detection of *mecA* and *nuc* genes in staphylococci by real-time multiplex polymerase chain reaction. *Diagn Microbiol Infect Dis* 2005; **51**: 13–17.
16. Martineau F, Picard FJ, Ke D *et al*. Development of a PCR assay for identification of staphylococci at genus and species levels. *J Clin Microbiol* 2001; **39**: 2541–2547.
17. Salo P, Ortqvist A, Leinonen M. Diagnosis of bacteremic pneumococcal pneumonia by amplification of pneumolysin gene fragment in serum. *J Infect Dis* 1995; **171**: 479–482.
18. Ke D, Bergeron MG. Molecular methods for rapid detection of group B streptococci. *Expert Rev Mol Diagn* 2001; **1**: 175–181.
19. Bryant PA, Li HY, Zaia A *et al*. Prospective study of a real-time PCR that is highly sensitive, specific, and clinically useful for diagnosis of meningococcal disease in children. *J Clin Microbiol* 2004; **42**: 2919–2925.
20. Greiner O, Day PJ, Altwegg M *et al*. Quantitative detection of *Moraxella catarrhalis* in nasopharyngeal secretions by real-time PCR. *J Clin Microbiol* 2003; **41**: 1386–1390.
21. Marty A, Greiner O, Day PJ *et al*. Detection of *Haemophilus influenzae* type b by real-time PCR. *J Clin Microbiol* 2004; **42**: 3813–3815.
22. Huijsdens XW, Linskens RK, Mak M *et al*. Quantification of bacteria adherent to gastrointestinal mucosa by real-time PCR. *J Clin Microbiol* 2002; **40**: 4423–4427.
23. Penders J, Vink C, Driessen C *et al*. Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR. *FEMS Microbiol Lett* 2005; **243**: 141–147.
24. Qin X, Emerson J, Stapp J *et al*. Use of real-time PCR with multiple targets to identify *Pseudomonas aeruginosa* and other nonfermenting gram-negative bacilli from patients with cystic fibrosis. *J Clin Microbiol* 2003; **41**: 4312–4317.
25. Wilson DA, Yen-Lieberman B, Reischl U *et al*. Detection of *Legionella pneumophila* by real-time PCR for the *mip* gene. *J Clin Microbiol* 2003; **41**: 3327–3330.
26. Greisen K, Loeffelholz M, Purohit A *et al*. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. *J Clin Microbiol* 1994; **32**: 335–351.
27. Rodriguez-Lazaro D, Hernandez M, Pla M. Simultaneous quantitative detection of *Listeria* spp. and *Listeria monocytogenes* using a duplex real-time PCR-based assay. *FEMS Microbiol Lett* 2004; **233**: 257–267.
28. Hoymans VY, Bosmans JM, Ursi D *et al*. Immunohistostaining assays for detection of *Chlamydia pneumoniae* in atherosclerotic arteries indicate cross-reactions with nonchlamydial plaque constituents. *J Clin Microbiol* 2004; **42**: 3219–3224.
29. Wang H, Kong F, Jelfs P *et al*. Simultaneous detection and identification of common cell culture contaminant and pathogenic mollicutes strains by reverse line blot hybridization. *Appl Environ Microbiol* 2004; **70**: 1483–1486.
30. Broccolo F, Scarpellini P, Locatelli G *et al*. Rapid diagnosis of mycobacterial infections and quantitation of *Mycobacterium tuberculosis* load by two real-time calibrated PCR assays. *J Clin Microbiol* 2003; **41**: 4565–4572.